Immunology, 1971, **20**, 693.

Identification by Density Separation of Antigen-specific Surface Receptors on the Progenitors of Antibody-producing Cells

R. M. Gorczynski, R. G. Miller and R. A. Phillips

Department of Medical Biophysics, University of Toronto, and Ontario Cancer Institute, Toronto, Ontario

(*Received* 28*th July* 1970)

Summary. Initiation of an immune response to sheep erythrocytes by the transplantation of spleen cells into irradiated recipients is thought to involve an interaction between two functionally different lymphoid cells. If such populations actually exist, it should be possible to separate them from each other and to study the properties of each type of cell. To this end, mouse spleen cells have been fractionated using equilibrium centrifugation in density gradients of Ficoll. Two functionally different populations of spleen cells were obtained. Neither population was active in initiating an immune response by itself on transplantation into irradiated mice, but mixtures of the two populations were. One population of spleen cells was also active when transplanted together with normal mouse bone marrow and the other population was active when mixed with thymus cells from normal mice. These results indicate that two different spleen cells must interact to initiate an immune response *in vivo*.

The specificity of the cell that synergizes with thymus cells was investigated further and shown to have antigen-specific receptors on its surface. These cells can be recognized by their ability to make rosettes with erythrocyte antigens. Thus, the 'background' rosette-forming cells found in unimmunized mice are actually progenitors of antibody-producing cells.

INTRODUCTION

The term 'antigen-sensitive unit' (ASU) may be defined as the minimum aggregate of cells that is active in initiating an immune response (Shearer, Cudkowicz and Priore, 1969). *In vitro* experiments studying the immune response of mice to various erythrocyte antigens indicate that three functionally different cells are required to form an ASU, two lymphoid cells and an accessory cell (Mosier, Fitch, Rowley and Davies, 1970; Haskill, Byrt and Marbrook, 1970; Shortman, Diener, Russell and Armstrong, 1970; Osoba, 1970). There is also evidence that similar cells comprise an ASU *in vivo*. Bone marrow-thymus synergism described by Claman (1969) and by Miller and Mitchell (1969) suggests the requirement for two lymphoid cells *in vivo*. Since the accessory cell required *in vitro* is radiation resistant (Roseman, 1969), its requirement *in vivo* using irradiated mice as recipients is difficult to confirm, but numerous indirect observations strongly implicate a

Correspondence: Dr R. A. Phillips, Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Ontario, Canada.

role for a radiation resistant phagocytic cell *in vivo* (Mitchison, 1969; Unanue and Cerottini, 1970).

For simplicity we will use the following nomenclature in this paper: The active cells in bone marrow or cells with properties like bone marrow cells will be called *B-cells*. The functional definition of a B-cell is a cell that interacts with thymus cells to initiate an immune response on transplantation into irradiated mice. Similarly, thymus cells and cells acting like thymus cells will be called *T-cells* and will be defined as any cells capable of interacting with added bone marrow cells to initiate an immune response on transplantation into irradiated recipients.

In this paper we describe experiments designed to study two features of this model for the initiation of immune responses *in vivo*. First, is the model applicable to tissues such as spleen which have ASU activity by themselves on transplantation into irradiated animals? Bone marrow-thymus synergism (Claman, 1969; Miller and Mitchell, 1969) and spleen-bone marrow synergism Cunningham, 1969; Talmage, Radovitch and Hemmingsen, 1969; Jacobson, L'Age-Stehr and Herzenberg, 1970) are consistent with the hypothesis of cellular interaction as is the observation of non-linearity of the dose-response curve for antibody-producing cells when increasing numbers of spleen cells are transplanted (Gregory and Lajtha, 1968; Groves, Lever and Makinodan, 1970; Talmage *et al.*, 1969). However, there is no direct evidence for the interaction *in vivo* of two spleen cells in the initiation of an immune response. A direct test of such an interaction should be possible by cell separation. If two or more functionally different cells are required, it should be possible to obtain two populations of cells from spleen which give ASU activity only when transplanted together into irradiated recipients. The data presented below show that two synergistic populations of cells can be obtained from suspensions of mouse spleen cells by fractionation on density gradients of Ficoll (Gorczynski, Miller and Phillips, 1970).

The second aspect of the model we have studied concerns the specificity of B-cells. That T-cells have antigen specificity has been shown in studies on bone marrow-thymus synergism (Miller and Mitchell, 1969), immunological memory (Talmage *et al.*, 1969; Cunningham, 1969) and immunological tolerance (Davies, 1969; Munro and Hunter, 1970; Mosier *et al.*, 1970). Miller and Mitchell (1970) have suggested that the B-cells in bone marrow–thymus synergism may be non-specific, even though they also demonstrated that antibody-producing cells are derived from B-cells and not T-cells (Nossal, Cunningham, Mitchell and Miller, 1968). However, in a previous communication we presented indirect evidence that B-cells have specific antigen receptors on their surface (Miller and Phillips, 1970). The experiments reported below confirm our hypothesis that the progenitors of antibody-producing cells, i.e. B-cells, have specificity.

MATERIALS AND METHODS

Mice

C3H/HeJ mice (purchased from The Jackson Laboratory, Bar Harbor, Maine) were used in all experiments. Males and females were used interchangeably. Mice were kept three to a cage and allowed free access to food and water.

Irradiation

All recipients were exposed to 950 rads of ionizing radiation using a ^{137}Cs irradiator at a

dose rate of 102 rads/min (Cunningham, Bruce and Webb, 1965) Cells were injected into irradiated recipients within 2 hours of irradiation.

Antigen

Sheep erythrocytes (SRBC) and horse erythrocytes (HRBC) were obtained weekly from Woodland Farms Limited, Guelph, Ontario. Before use, either as antigen or as indicator in assays for antibody-producing cells, they were washed three times in phosphate-buffered saline (PBS).

Preparation of cell suspensions

For suspensions of spleen or thymus, the tissue was cut into fine pieces with scissors and gently pressed through a stainless steel screen (60 mesh) with the blunt end of the plunger from a tuberculin syringe. During this procedure the stainless steel mesh was repeatedly dipped into the buffer solution used for the density gradient to remove cells released from the small pieces of tissue. Large clumps of cells and debris were removed by allowing them to settle for 15 minutes and then removing the supernatant which contained mostly single cells or only small clumps of cells. With this method of preparing cell suspensions 90–95 per cent of the nucleated cells take up the vital dye fluorescein diacetate (Rotman and Papermaster, 1966).

Bone marrow suspensions were made by gently squirting PBS through the femur. With this procedure the bone marrow came out as a solid plug. Gentle aspiration with a 10 ml pipette released the majority of the nucleated cells, maintaining viability at 90 per cent or better as assessed by the uptake of fluorescein diacetate.

Density gradient separation

Full details of the method using isotonic Ficoll density gradients have been presented elsewhere (Gorczynski *et al.*, 1970). All separations were performed at pH 5·5. Two modifications were made in the previously described method. The first was the use in some experiments of a zonal centrifuge in order to process large numbers of cells. Using an A-XII zonal rotor (with an MSE model 6L centrifuge), up to 7×10^9 mouse cells could be processed. The loading procedure was as follows: The cells were mixed with the light density Ficoll medium. High density Ficoll medium was pumped into the low density reservoir and thoroughly mixed with the material therein. This mixture was then pumped into the zonal rotor at twice the rate at which new medium was being pumped into the low density reservoir. The gradient was generated continuously over a 15 minute period while the rotor was spinning at 500 rev/min. The rapid generation of the gradient together with the surging of the pumps led to some non-linearity in the gradients. Corrections for this were made according to the method suggested by Shortman (1968).

We routinely used centrifugation times of 45 minutes at rotor speeds of 5250 rev/min. This speed gives a force of 5700 *g* at the outer edge of the rotor but only 2000 *g* at the inner edge. Control experiments in which the distribution of numerous cell types were compared to the density profiles obtained with the smaller gradients described earlier (Gorczynski *et al.*, 1970) showed no loss of resolution with the zonal centrifuge.

To collect fractions at the end of a separation, 100 ml of a dense Ficoll solution were pumped to the outer edge of the rotor followed by 60 per cent sucrose to displace the gradient containing the cells. The dense cushion prevented direct contact and mixing of the gradient containing the cells with the dense sucrose used to displace the gradient. In most

experiments twenty-six fractions of 50 ml each were collected. To concentrate the cells in each fraction, the fraction was diluted 10- to 15-fold (according to the density of the medium) in buffer and the cells centrifuged at 600 ***g*** for 15 minutes in siliconized 250 ml glass bottles. With this procedure, we routinely obtained 30–50 per cent recovery of nucleated cells.

The second modification of the separation procedure was the use of step gradients. After the completion of preliminary experiments to define the densities of the various cell types, it was only necessary to examine the cells within particular density intervals. Solutions of the desired densities were layered into a small 30 ml centrifuge tube in order of decreasing density, cells having been introduced into each of the solutions at a concentration of 5×10^6/ml, and the tube centrifuged for 60 minutes at 3800 ***g***. The cells form bands at the interfaces between the solutions of different density. Fractions were collected by withdrawing the different layers using a small pipette.

ASU activity

ASU activity in unfractionated tissues or in fractions obtained after centrifugation was assayed by mixing the cells with 1×10^8 SRBC and injecting the mixture into lethally irradiated recipients. In some experiments designed to determine the distributions of B- and T-cells, thymus cells (5×10^7 per recipient mouse) or bone marrow cells (5×10^6 per recipient mouse) respectively, were also mixed in with the fractions. Eight days after transplantation, the spleen from each survivor was suspended in 1 ml of PBS and assayed for its content of 19S plaque-forming cells (PFC) using the assay of Jerne, Nordin and Henry (1963).

Rosette formation

Two different methods for forming rosettes were used. In the early experiments, we followed the procedure described by Shearer and Cudkowicz (1968): Cell suspensions containing 10^6 nucleated cells/ml and 8×10^6 SRBC/ml were incubated for 85 minutes at 37° in a CO_2 incubator. This method routinely yielded 300 rosettes/10^6 nucleated cells. In later experiments, we found that a higher yield of rosettes could be obtained with the method described by Osoba (1970): 2×10^7 spleen cells were mixed with an eight-fold excess of sheep or horse erythrocytes. The suspension was centrifuged at 20 ***g*** for 15 minutes and the pellet incubated for 15 minutes at 4°. The yield of rosettes by this method was routinely somewhat higher (500 rosettes/10^6 nucleated spleen cells for SRBC) than with the method described by Shearer and Cudkowicz (1968).

RESULTS

Presence of B- and T-cells in mouse spleen

Studies on the sedimentation properties of ASU indicated that one of the active cells, the B-cell, has specific surface receptors and can be recognized by its ability to form rosettes with the stimulating antigen (Miller and Phillips, 1970). To determine whether or not useful separation of B-cells and T-cells could be achieved by fractionation of suspensions of spleen cells on gradients of Ficoll, the density profiles for ASU and rosette-forming cells (RFC) were compared. Spleen cells, obtained from unimmunized mice, were separated on a Ficoll density gradient in a zonal centrifuge, and each fraction was assayed for its content of nucleated cells, RFC, and ASU activity. The results of one such experiment are

shown in Fig. 1. The distribution of nucleated cells (upper panel) is identical to the distribution reported earlier (Gorczynski *et al.*, 1970) in which the separation was performed in a 30 ml centrifuge tube rather than a zonal centrifuge. The distributions of ASU activity and RFC are given in the bottom panel of Fig. 1. The distribution of ASU is broad and has two definite peaks, at densities of 1·054 and 1·076 g/cm^3. The RFC, however, appear to be a single homogeneous population with a modal density of 1·065 g/cm^3, though there may be a minor population of RFC at a density of approximately 1·055 g/cm^3.

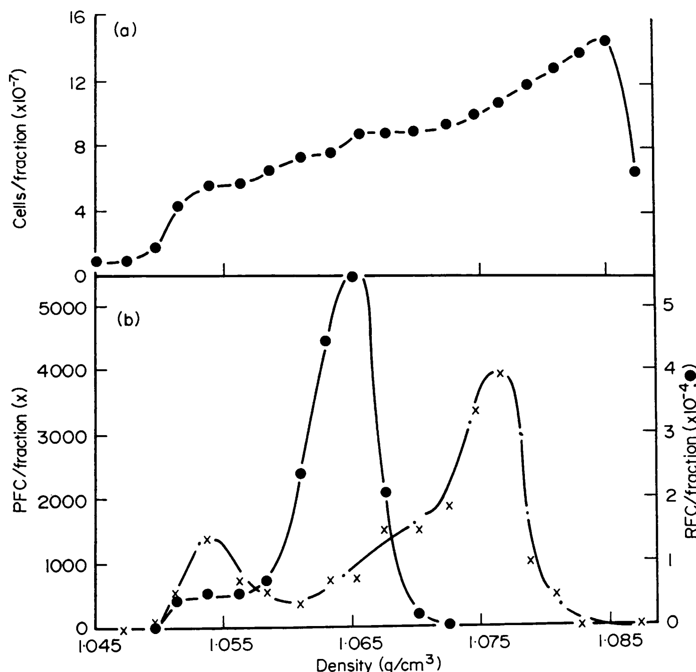

FIG. 1. ASU activity and RFC distribution from unimmunized C3H spleen cells fractionated by equilibrium flotation in Ficoll using a zonal centrifuge. (a) Nucleated cell profile. Recovery was 90 per cent. (b) ×, ASU distribution as measured by the number of PFC obtained on transplantation of 1/20 of the cells in each fraction (mixed with 10^8 SRBC) into irradiated recipients. A total of 2×10^9 nucleated cells were processed. Thus, each recipient mouse received cells of a particular density equal to the number in 10^8 unfractionated cells. The recovery of activity was only 20–30 per cent of the ASU activity expected in 2×10^8 cells. ●, RFC distribution. Recovery was 40 per cent of the input as calculated from the RFC frequency in an unfractionated control.

If the B-cells required for initiation of the immune response can also form rosettes, the results of Fig. 1 can only be explained by assuming that the T-cell in the spleen exists in two density states, one of low density and one of high density. The two peaks of ASU activity would then correspond to the regions of overlap of the two T-cell distributions and the RFC distribution. Two types of experiments were done to determine the distributions of B- and T-cells in mouse spleen.

The first experiment was to confirm that two interacting cells exist in mouse spleen. Our working hypothesis predicted that if spleen cells from either end of the gradient (where there should be many T-cells) were added to the region containing RFC, the

mixture should be active in the ASU assay. Table 1 gives typical results from one of several experiments testing this prediction. Spleen cells were separated on a step gradient and two fractions were selected: one contained cells in the density interval 1·060–1·071 g/cm^3 and the other 1·080–1·095 g/cm^3. The first interval contains most of the RFC and should contain mainly B-cells, and the latter should contain mainly T-cells. Cells from either fraction alone gave little activity when transplanted alone, but a mixture of the two gave a response equivalent to 1×10^7 unfractionated spleen cells. These data are consistent with the hypothesis that spleen contains two populations of cells each of which are required to initiate an immune response *in vivo*.

A more precise estimate of the actual distributions of B- and T-cells in mouse spleen can be obtained by fractionating spleen, adding bone marrow cells or thymus cells to each fraction, and assaying for ASU activity. If thymus cells are added to each fraction, they will interact with the B-cells in that fraction to give an indication of the actual content of B-cells in that fraction. Similarly, addition of bone marrow cells should give an indication of the actual T-cell distribution.

Table 1
Synergism between low and high density spleen cells

Group	Source of cells*	No. cells injected	PFC/spleen
A	$1{\cdot}060 < \rho < 1{\cdot}071$ g/cm^3	8×10^6†	5 (3–9)
B	$1{\cdot}071 < \rho < 1{\cdot}080$	5×10^6‡	76 (62–93)
C	$1{\cdot}080 < \rho < 1{\cdot}095$	8×10^6§	13 (10–16)
D	A+C	4×10^6 A + 4×10^6 C	160 (110–230)
E	Unfractionated spleen	1×10^7	110 (100–130)
F	Unfractionated spleen	5×10^6	29 (25–35)

* Fractionated cells obtained by separation of $1{\cdot}5 \times 10^8$ spleen cells on a step gradient.
† Equivalent to the number of cells of that density found in $3{\cdot}5 \times 10^7$ unfractionated spleen cells.
‡ Equivalent to the number of cells of that density found in $1{\cdot}1 \times 10^7$ unfractionated spleen cells.
§ Equivalent to the number of cells of that density found in $2{\cdot}5 \times 10^7$ unfractionated spleen cells.
¶ Geometric mean of PFC / spleen in recipients. Numbers in parentheses are 95 per cent confidence limits.

The results of two replicate experiments are summarized in Table 2. For comparison of the various groups, the results of the experiment in Table 2(a) are also presented graphically in Fig. 2. The density separation was performed exactly as in Fig. 1, but to restrict the experiment to manageable proportions, fractions were pooled, generally in groups of three. Each pool was then divided into three equal aliquots. The first aliquot was mixed only with SRBC to measure ASU activity. The second aliquot was mixed with SRBC and bone marrow cells to determine the T-cell distribution. The third was mixed with SRBC and thymus cells to determine the B-cell distribution. Groups of irradiated mice were injected with cells from all aliquots. Each mouse received 1/100th of the cells in the pool from the gradient (equivalent to the cells of that density found in $3{\cdot}2 \times 10^7$ unfractionated spleen cells) plus 10^8 SRBC alone, or 10^8 SRBC plus either 5×10^6 bone marrow cells or 5×10^7 thymus cells.

Fig. 2 shows the data of Table 2 plotted as a histogram. The width of each bar indicates the density interval from which the injected spleen cells were taken, and the area of the bar is proportional to the ASU activity in that pool. The distributions of both nucleated cells (Fig. 2a) and ASU activity (Fig. 2b) in spleen fractions alone are similar to those shown in Fig. 1, though there is probably a significantly higher proportion of non-viable

TABLE 2(a)

SYNERGISM OF SPLEEN FRACTIONS WITH BONE MARROW AND THYMUS CELLS

Density interval	ASU activity (PFC/spleen)*		
	Fraction alone†	Fraction + Bone marrow‡	Fraction + thymus§
$\rho < 1{\cdot}051$ g/cm^3	1 (0–1) (6)¶	77 (70–86) (6)¶	N.S. (0)¶
$1{\cdot}051 < \rho < 1{\cdot}060$	87 (77–98) (6)¶	115 (79–170) (5)¶	98 (85–120) (7)¶
$1{\cdot}060 < \rho < 1{\cdot}065$	16 (13–20) (8)¶	30 (23–29) (7)¶	186 (145–250) (10)¶
$1{\cdot}065 < \rho < 1{\cdot}072$	96 (68–140) (7)¶	101 (83–130) (7)¶	154 (130–180) (8)¶
$1{\cdot}072 < \rho < 1{\cdot}080$	240 (200–285) (9)¶	187 (148–240) (6)¶	228 (180–280) (8)¶
$1{\cdot}080 < \rho < 1{\cdot}083$	36 (32–40) (6)¶	237 (190–295) (7)¶	N.S. (0)¶
$1{\cdot}083 < \rho < 1{\cdot}090$	N.S. (0)¶	894 (600–1130) (10)¶	N.S. (0)¶
$1{\cdot}090 < \rho < 1{\cdot}093$	15 (12–19) (10)¶	595 (440–780) (8)¶	21 (19–23) (6)¶
$1{\cdot}093 < \rho < 1{\cdot}096$	5 (3–8) (9)¶	21 (19–23) (6)¶	N.S. (0)¶

* Each entry represents the geometric mean of the PFC per recipient spleen for the group. Values in parentheses give 95 per cent confidence limits.

† Each mouse received 1/100th of the cells in the density fraction indicated together with 10^8 SRBC.

‡ Each mouse also received 5×10^6 bone marrow cells.

§ Each mouse also received 5×10^7 thymus cells.

¶ Number of mice surviving to the day of assay (day 8).

N.S. No survivors.

TABLE 2(b)

SYNERGISM OF SPLEEN FRACTIONS WITH BONE MARROW AND THYMUS CELLS

Density interval	ASU activity (PFC/spleen)*		
	Fraction alone†	Fraction + bone marrow‡	Fraction + thymus§
$\rho < 1{\cdot}057$ g/cm^3	76 (58–101) (9)¶	280 (179–396) (10)¶	78 (51–132) (9)¶
$1{\cdot}057 < \rho < 1{\cdot}063$	12 (5–32) (8)¶	78 (51–132) (10)¶	100 (54–201) (10)¶
$1{\cdot}063 < \rho < 1{\cdot}068$	44 (21–86) (10)¶	264 (190–361) (9)¶	198 (170–251) (8)¶
$1{\cdot}068 < \rho < 1{\cdot}072$	54 (36–91) (8)¶	282 (181–402) (10)¶	76 (56–103) (8)¶
$1{\cdot}072 < \rho < 1{\cdot}073$	100 (111–269) (10)¶	208 (120–269) (10)¶	112 (71–181) (10)¶
$1{\cdot}073 < \rho < 1{\cdot}076$	340 (201–576) (9)¶	348 (232–510) (10)¶	340 (220–543) (8)¶
$1{\cdot}076 < \rho < 1{\cdot}082$	32 (22–53) (10)¶	84 (52–143) (10)¶	40 (21–76) (8)¶
$1{\cdot}082 < \rho < 1{\cdot}086$	30 (17–61) (7)¶	588 (332–971) (10)¶	31 (15–73) (6)¶
$1{\cdot}086 < \rho < 1{\cdot}094$	30 (21–52) (5)¶	240 (170–396) (10)¶	N.S. (0)¶
$1{\cdot}094 < \rho < 1{\cdot}098$	8 (4–23) (10)¶	84 (51–158) (9)¶	N.S. (0)¶

Footnotes are identical to those in Table 2(a).

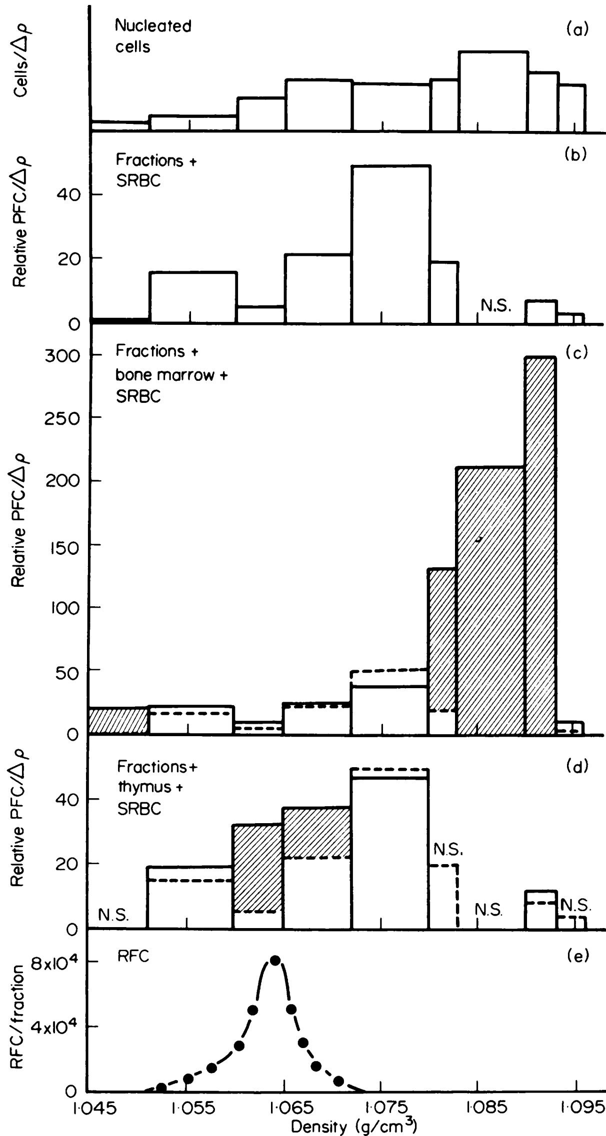

FIG. 2. ASU activity against SRBC in spleen fractions separated by equilibrium flotation in Ficoll on a zonal centrifuge. Pooled fractions were assayed alone (b), mixed with 5×10^6 bone marrow cells (c), and with 5×10^7 thymus cells (d). The area under the curves represents the relative number of PFC obtained in each density interval. Curve (a) shows the nucleated cell profile and curve (e) the RFC distribution. In curves (c) and (d) the control PFC distribution (given by curve b) is represented by a broken line. Areas of significant synergism are indicated by cross-hatching. N.S. indicates no survivors. The same data are presented in tabular form in Table 2(a).

cells (as judged by their high density (Gorczynski *et al.*, 1970)). In Fig. 2(c) and 2(d), the activity of the fraction alone, i.e. the distribution shown in Fig. 2(b), is given by a dashed line. Extra activity resulting from synergism with bone marrow or thymus is indicated by cross-hatching. As predicted, the fractions that synergize with bone marrow cells are at the two extremes of the gradient (Fig. 2c). By definition these two fractions contain

T-cells. The fractions that synergize with added thymus cells (Fig. 2d) appear to be the same as those that contain RFC (Fig. 2e).

Identification and specificity of B-cells

In the previous section it was shown that fractions containing B-cells also contain RFC. It should be possible to use the rosette-forming capacity of RFC to test directly whether or not B-cells are also RFC. Work of Moav and Harris (1968), using density separations on gradients of bovine serum albumin, showed that RFC having sheep erythrocytes bound to their surface banded at a density close to that of SRBC alone. Preliminary experiments showed that SRBC banded in Ficoll gradients at a density of 1·080 g/cm^3, well removed from the RFC distribution, which peaks at a density of 1·065 g/cm ^{3}. If RFC are B-cells, a shift in their distribution should also give a shift in the fractions that can interact with thymus cells in a transplantation assay. Such an experiment would also give information on the specificity of B-cells. If specific, the position of B-cell activity in the gradient should shift only for the kind of erythrocyte used to form the rosette before separation. A series of experiments was performed to test the effect of preformation of rosettes on B-cell distributions.

TABLE 3

SYNERGISM OF UNFRACTIONATED SPLEEN CELLS WITH THYMUS CELLS

Group	Cells injected*	PFC to HRBC† per ½ spleen	PFC to SRBC† per ½ spleen
1	5×10^6 unfractionated spleen cells	3 (1–5)	6 (4–11)
2	$4{\cdot}5 \times 10^7$ thymus cells	0 (0–0)	0 (0–0)
3	5×10^6 unfractionated spleen cells $4{\cdot}5 \times 10^7$ thymus cells	88 (73–106)	83 (64–108)

* All mice received 10^8 SRBC, 10^8 HRBC, and nucleated cells as indicated.
† Geometric mean of the number of PFC/½ spleen obtained on transplantation. The numbers in parentheses are the 95 per cent confidence limits.

In these experiments 2×10^7 spleen cells were fractionated on each of three identical step gradients prepared with Ficoll of five different densities. After separation the corresponding fractions from the separate gradients were pooled. Thus, each pool contained all the cells of that density interval found in 6×10^7 spleen cells. Each irradiated recipient received 1/12 of the cells in the fraction (i.e. the number of cells in that density interval to be found in 5×10^6 unfractionated spleen cells) mixed with 5×10^7 thymus cells, 10^8 SRBC and 10^8 HRBC. This number of spleen cells is substantially lower than for the experiments of Figs 1 and 2 and was found to be active on transplantation only with the addition of thymus cells (see Table 3). Thus, the activity distribution found on the gradient should be a direct representation of the B-cell distribution. Columns 3 and 4 of Table 4 show the results obtained when spleen cells from unimmunized mice were fractionated and assayed for B-cells both for SRBC and HRBC. As expected, the distributions of B-cells for both types of erythrocytes were identical, and activity was found only in the regions known to contain the majority of the RFC, i.e. the density interval 1·062–1·070 g/cm^3. (see Figs 1b and 2e). Next, horse erythrocytes were used to form rosettes prior to separation. Fig. 3(a) shows that the distribution of rosettes, formed with HRBC before separation, has shifted to the density interval near 1·080 g/cm^3. When each of the fractions from this experiment was assayed for B-cell activity, it was observed (Fig. 3b,

TABLE 4

EFFECT OF PREFORMATION OF ROSETTES ON THE DENSITY PROFILE OF B-CELLS

Fraction	Density interval	PFC/½ spleen§					
		Control*		Preformed HRBC-RFC†		Preformed SRBC-RFC‡	
		SRBC	HRBC	SRBC	HRBC	SRBC	HRBC
A	$1.050 < \rho < 1.062$	1 (1–3)	4 (2–7)	1 (1–2)	1 (0–1)	4 (2–7)	2 (1–4)
B	$1.062 < \rho < 1.070$	75 (60–94)	69 (55–86)	55 (38–78)	0 (0–1)	2 (1–5)	58 (43–78)
C	$1.070 < \rho < 1.078$	0 (0–1)	1 (0–1)	4 (2–8)	0 (0–1)	1 (0–2)	0 (0–0)
D	$1.078 < \rho < 1.086$	1 (1–2)	0 (0–1)	2 (1–4)	59 (51–68)	74 (60–90)	0 (0–1)
E	$1.086 < \rho < 1.094$	0 (0–0)	0 (0–0)	0 (0–1)	0 (0–1)	2 (1–5)	1 (0–1)

* Fractionation of normal spleen with no prior rosette formation.
† HRBC used to form rosettes prior to separation.
‡ SRBC used to form rosettes prior to separation.
§ Each spleen was suspended in 1 ml of PBS; 0·5 ml was assayed for PFC for SRBC and 0·5 ml for PFC specific for HRBC. The geometric means of the PFC/½ spleen are shown in the Table. The values in parentheses are the 95 per cent confidence limits.

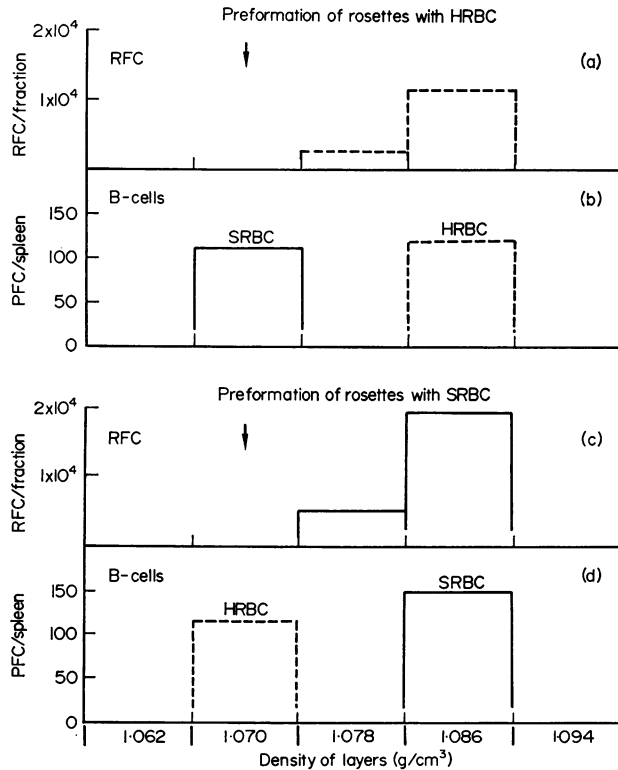

FIG. 3. Effect of preformation of rosettes on the density profile of B-cells. Panels (a) and (b) give the results obtained when rosettes to horse erythrocytes were formed before separation and fractions were tested for B-cell activity to SRBC and HRBC. In (a), the arrow indicates the density interval in which all the RFC to either SRBC or HRBC are found when rosettes are not preformed. The broken line shows the distribution of HRBC rosettes. Panel (b) shows the B-cell activity for SRBC (solid line) and HRBC broken line). See text for details of the assay. Note that the B-cells to HRBC have moved with the HRBC rosettes. Panels (c) and (d) give the results of a similar experiment where rosettes were preformed to SBRC instead of HRBC. Here the B-cells to SRBC have moved. The same data are presented in tabular form in Table 4.

Table 4, columns 5 and 6) that the activity for HRBC had shifted to the interval containing the majority of rosettes specific for HRBC while the activity for SRBC remained in the normal position (indicated by the arrow in Fig. 3a). Fig. 3(c) and 3(d) give the results of a reciprocal experiment in which SRBC were used to form rosettes prior to separation. As before, rosette formation shifted RFC specific to SRBC to a higher density (Fig. 3c) and also shifted the B-cell activity specific for SRBC to the same region (Fig. 3d and Table 4, columns 7 and 8). The B-cell activity specific for HRBC remained unchanged from the control. Similar results were obtained on each of the six occasions that this experiment was repeated.

DISCUSSION

Two other groups of investigators have separated mouse spleen cells on gradients of bovine serum albumin (BSA) and assayed fractions *in vivo* for ASU activity specific for SRBC; both groups observed two peaks of ASU activity similar to the peaks shown here (Fig. 1). Haskill *et al.* (1970) detected ASU at densities of 1·064 and 1·069 g/cm^3, while Shortman *et al.* (1970) found activity at densities of 1·068 and 1·075 g/cm^3. In contrast, the peaks of activity on Ficoll gradients (Fig. 1) are at densities of 1·054 and 1·076 g/cm^3. Thus, one of the peaks corresponds to the dense peak observed by Shortman *et al.* (1970), but the low density peak in Ficoll has no apparent counterpart in BSA gradients. Some possible causes for discrepancies in density values obtained in Ficoll and BSA have been previously discussed by Gorczynski *et al.* (1970). Unfortunately, neither Haskill *et al.* (1970) nor Shortman *et al.* (1970) tested the ability of various fractions to interact with added bone marrow or thymus cells. A lack of these data makes it impossible to determine whether or not the peaks of activity on gradients of BSA arise from overlapping cell populations analogous to the situation for Ficoll gradients.

The present data provide a direct confirmation of the hypothesis that two different spleen cells must interact to initiate an immune response *in vivo*. In addition, these data, coupled with those of earlier studies, allow one to make several definite statements about the properties of one of the required cells, the B-cells:

(1) B-cells are small cells that do not secrete antibody. Sedimentation analysis of B-cells (Miller and Phillips, 1970) and antibody-producing cells (Phillips and Miller, 1970) show that these cells sediment at different rates. Density analysis of antibody-producing cells (Gorczynski *et al.*, 1970) shows that they band at a different density than B-cells.

(2) B-cells specifically recognize the antigen to which they respond. The data in Fig. 3 clearly show that B-cells can form rosettes, i.e. they have specific surface receptors that bind the antigen to which they respond. These data are also consistent with the data of Ada and Byrt (1969) that the immune response can be specifically suppressed with radioactive antigen. Presumably, in their experiments, radioactive antigen binds specifically to B-cells causing a localization of radiation that inactivates them.

(3) B-cells are the progenitors of antibody-producing cells. Using chromosome markers, Nossal *et al.* (1968) showed that the cells that interact with thymus are the progenitors of antibody-producing cells. That is, in terms of our model, B-cells are the progenitors of antibody-producing cells.

Using a cell culture assay and other methods of cell separation, Osoba (1970) has carried out similar experiments to those presented here. His data also show that rosette-forming cells are one of the cells required to initiate a response *in vitro*.

The nature and function of the second class of cells, the T-cells, remain a mystery. They appear to exist in two subpopulations, one of high density and one of low density. Although there is evidence that T-cells have specificity (Miller and Mitchell, 1969), the origin of this specificity is not clear. In contrast to B-cells, whose specificity exists prior to the introduction of antigen, the pre-existence of specificity in T-cells is not proven. The present data indicate that T-cells do not seem to have specific antigen receptors on their surface; in the density intervals most active for T-cells we could detect no cells capable of forming rosettes. In primed animals T-cells appear to be specific (Cunningham, 1969; Talmage *et al.*, 1969) but T-cells in such animals may have acquired specificity as a result of their previous exposure to antigen. Nevertheless, the demonstration of specificity for B-cells would seem to rule out information transfer as a role for T-cells (Adler, Fishman and Dray, 1966; Miller and Mitchell, 1970). Perhaps T-cells recognize carrier antigens and function to position the antigen in a conformation suitable to trigger the differentiation and proliferation of B-cells (Bretscher and Cohn, 1968). Alternatively, T-cells may elaborate a specific factor involved in the regulation of B-cells (Kennedy, Treadwell and Lennox, 1970; Dumonde, Wolstencroft, Panayi, Matthew, Morley and Howson, 1969). Hopefully, the ability to separate B-cells and T-cells from each other will allow a more careful study of the precise function of T-cells and of their interactions with B-cells.

ACKNOWLEDGMENTS

Excellent technical assistance was provided by Mr R. Kuba, Miss Heather Mooney and Mrs Judy Madrus.

This research was supported by the National Cancer Institute of Canada, the Donner Canadian Foundation, and the Medical Research Council (grant MA-3017). R.M.G. is supported by a Commonwealth Scholarship.

REFERENCES

ADA, G. L. and BYRT, P. (1969). 'Specific inactivation of antigen-reactive cells with I^{125}-labelled antigen.' *Nature (Lond.)*, **222**, 1291.

ADLER, F. L., FISHMAN, M. and DRAY, S. (1966). 'Antibody formation initiated *in vitro*. III. Antibody formation and allotypic specificity directed by ribonucleic acid from peritoneal exudate cells.' *J. Immunol.*, **97**, 554.

BRETSCHER, P. D. and COHN, M. (1968). 'Minimal model for the mechanism of antibody induction and paralysis by antigen.' *Nature (Lond.)*, **220**, 444.

CLAMAN, H. N. (1969). 'Immunologic complementation between thymus and marrow cells—a model for the two-cell theory of immunocompetence.' *Transpl. Rev.*, **1**, 92.

CUNNINGHAM, A. J. (1969). 'Studies on the cellular basis of IgM immunological memory. The induction of antibody formation in bone marrow cells by primed spleen cells.' *Immunology*, **17**, 933.

CUNNINGHAM, J. R., BRUCE, W. R. and WEBB, H. P. (1965). 'A convenient ^{137}Cs unit for irradiating cell suspensions and small laboratory animals.' *Phys. Med. Biol.*, **10**, 381.

DAVIES, A. J. S. (1969). 'The thymus and the cellular basis of immunity.' *Transpl. Rev.*, **1**, 43.

DUMONDE, D. C., WOLSTENCROFT, R. A., PANAYI, G. S., MATTHEW, M., MORLEY, J. and HOWSON, W. T. (1969). 'Lymphokines: Non-antibody mediators of cellular immunity generated by lymphocyte action.' *Nature (Lond.)*, **224**, 38.

GORCZYNSKI, R. M., MILLER, R. G. and PHILLIPS, R. A. (1970). 'Homogeneity of antibody-producing cells as analysed by their buoyant density in gradients of Ficoll.' *Immunology* **19**, 817.

GREGORY, C. J. and LAJTHA, L. G. (1968). 'Kinetic study of the production of antibody-forming cells from their precursors.' *Nature (Lond.)*, **218**, 1079.

GROVES, D. L., LEVER, W. E. and MAKINODAN, T. (1970). 'A model for the interaction of cell types in the generation of hemolytic plaque-forming cells.' *J. Immunol.*, **104**, 148.

HASKILL, J. S., BYRT, P. and MARBROOK, J. (1970). '*In vitro* and *in vivo* studies of the immune response to sheep erythrocytes using partially purified cell preparations.' *J. exp. Med.*, **131**, 57.

JACOBSON, E. B., L'AGE-STEHR, J. and HERZENBERG, L. A. (1970). 'Immunological memory in mice. II. Cell interactions in the secondary immune response studied by means of immunoglobulin allotype markers.' *J. exp. Med.*, **131**, 1109.

JERNE, N. K., NORDIN, A. A. and HENRY, C. (1963). 'The agar plaque technique for recognizing antibody-

producing cells.' *Cell Bound Antibodies* (Ed. by B. Amos and H. Koprowski), p. 109. Wistar Institute Press, Philadelphia.

KENNEDY, J. C., TREADWELL, P. E. and LENNOX, E. S. (1970). 'Antigen-specific synergism in the immune response of irradiated mice given marrow cells or peritoneal cavity cells or extracts.' *J. exp. Med.*, **132**, 353.

MILLER, J. F. A. P. and MITCHELL, G. F. (1969). 'Thymus and antigen-reactive cells.' *Transpl. Rev.*, **1**, 3.

MILLER, J. F. A. P. and MITCHELL, G. F. (1970). 'Cell to cell interaction in the immune response. V. Target cells for tolerance induction.' *J. exp. Med.*, **131**, 675.

MILLER, R. G. and PHILLIPS, R. A. (1970). 'Sedimentation analysis of the cells in mice required to initiate an *in vivo* immune response to sheep erythrocytes.' *Proc. Soc. exp. Biol.* (*N.Y.*), **135**, 63.

MITCHISON, N. A. (1969). 'The immunogenic capacity of antigen taken up by peritoneal exudate cells.' *Immunology*, **16**, 1.

MOAV, N. and HARRIS, T. N. (1968). 'Collection of rosette-forming spleen cells by BSA density gradient centrifugation.' *Proc. Soc. exp. Biol.* (*N.Y.*), **128**, 407.

MOSIER, D. E., FITCH, F. W., ROWLEY, D. A. and DAVIES, A. J. S. (1970). 'Cellular deficiency in thymectomized mice.' *Nature* (*Lond.*), **225**, 276.

MUNRO, A. and HUNTER, P. (1970). '*In vitro* reconstitution of the immune response to thymus-deprived mice to sheep red blood cells.' *Nature* (*Lond.*), **225**, 277.

NOSSAL, G. J. V., CUNNINGHAM, A., MITCHELL, G. F. and MILLER, J. F. A. P. (1968). 'Cell to cell interaction in the immune response. III. Chromosomal marker analysis of single antibody-forming cells in reconstituted, irradiated or thymectomized mice.' *J. exp. Med.*, **128**, 839.

OSOBA, D. (1970). 'Some physiological and radiobiological properties of immunologically reactive mouse spleen cells.' *J. exp. Med.*, **132**, 368.

PHILLIPS, R. A. and MILLER, R. G. (1970). 'Antibody-producing cells: analysis and purification by velocity sedimentation.' *Cell Tissue Kinet.*, **3**, 263.

ROSEMAN, J. (1969). 'X-ray resistant cell required for the induction of *in vitro* antibody formation.' *Science*, **165**, 1125.

ROTMAN, B. and PAPERMASTER, B. W. (1966). 'Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters.' *Proc. nat. Acad. Sci.* (*Wash.*), **55**, 134.

SHEARER, G. M. and CUDKOWICZ, G. (1968). 'Cluster formation *in vitro* by mouse spleen cells and sheep erythrocytes.' *J. Immunol.*, **101**, 1266.

SHEARER, G. M., CUDKOWICZ, G. and PRIORE, R. L. (1969). 'Cellular differentiation of the immune system of mice. II. Frequency of unipotent splenic antigen-sensitive units after immunization with sheep erythrocytes.' *J. exp. Med.*, **129**, 185.

SHORTMAN, K. (1968). 'The separation of different cell classes from lymphoid organs. II. The purification and analysis of lymphocyte populations by equilibrium density gradient centrifugation.' *Aust. J. exp Biol. med. Sci.*, **46**, 375.

SHORTMAN, K., DIENER, E., RUSSELL, P. and ARMSTRONG, W. D. (1970). 'The role of non-lymphoid accessory cells in the immune response to different antigens.' *J. exp. Med.*, **131**, 461.

TALMAGE, D. W., RADOVITCH, J. and HEMMINGSEN, H. (1969). 'Cell interaction in antibody synthesis.' *Heterospecific Genome Interaction* (Ed. by V. Defendi). Wistar Institute Press, Philadelphia.

UNANUE, E. R. and CEROTTINI, J. C. (1970). 'The immunogenicity of antigen bound to the plasma membrane of macrophages.' *J. exp. Med.*, **131**, 711.